A droplet-based microfluidic platform for on-chip viral-fusion studies

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CHAPTER 6

Summary and perspective

The influenza virus is considered a major threat to public health. It spreads around the world in a seasonal cycle and a new pandemic could potentially arise due to the high genetic adaptability of the virus. With the aim of screening new anti-viral drugs in a fast and high-throughput manner, I have described in this thesis the combination of microfluidics using microdroplets as reaction chambers with fluorescence imaging techniques to develop a lab-on-a-chip platform that allows the monitoring of viral fusion events under different conditions and the characterization of the fusion kinetics. As shown in this thesis, these microdroplets can be generated by printing microdroplet arrays on a glass support or, alternatively, by using an emulsion droplet system. In each microdroplet, viral fusion can be triggered by lowering the pH inside the reaction chamber, resembling the same conditions encountered by the virus in endosomes. This thesis presents new developments in on-chip aqueous chemistry with applications in virus detection and neutralization assays.

In **chapter 1** I provide background and a broader academic framework by reviewing recent advances in the application of droplet microfluidic-chip-based technologies to bioengineering, biochemistry, chemical synthesis, and nanotechnology. We deal with topics such as single-cell analysis, small-scale cell cultures, *in-droplet* chemical synthesis, high-throughput drug screening, and fabrication of nano-devices. Additionally, I discuss emerging research directions and, in particular, propose approaches for the future use of droplet-microfluidics in virology.

Arrays of supported lipid bilayers (SLB) provide enormous potential for drug development and multiplexed biochemical research, but they are difficult to prepare for viral fusion studies because the fusion process requires a relatively complex bilayer composition as well as adequate space between the bilayer and the support. Establishing supported lipid bilayers with a biologically relevant composition, including transmembrane proteins and various classes of lipids, is a significant challenge. The high mobility of membrane components, which is ensured in the proposed platform, is very important in processes such as viral fusion where mobility and structural rearrangements of membrane proteins are needed. In **chapter 2** of this

thesis, we describe a generic and facile approach to the production of fluid polymer-supported lipid bilayers that allows for the incorporation of a wide variety of lipids and transmembrane proteins. The method is based on the formation of a polymer brush displaying lipid groups, followed by spin-coating of membrane lipids. Subsequently, transmembrane proteins are incorporated by the fusion of proteoliposomes with the bilayer. Additionally, several applications of the technology are also described; these include the single-molecule tracking of the incorporated transmembrane proteins into a bilayer, the visualization of the fusion of individual, membrane-enveloped viruses with a supported membrane, and antibody inhibition tests. Our results suggest that the membrane properties are consistent with those found in physiologically relevant conditions. This demonstrates the wide applicability of our approach to synthetic biology, which utilizes lab-on-a-chip technology combined with biophysical and pharmaceutical studies. As an example, we used this platform to perform a single-particle viral fusion assay on a chip, and this technology was subsequently applied to quantify the effectiveness of an antiviral antibody.

In **chapter 3**, we address a major challenge facing the development of droplet-based viral fusion assays: controlling the acidity of the droplet, independent of the concentrations of other reagents. This challenge is a common one in droplet-based chemistry: how to introduce new reactants into an aqueous droplet that exists within an oil phase. Our proposed approach relies on the use of reactants (in our case an acid) that are soluble in both the aqueous and oil phases. Central to our microfluidic approach are the solubility, diffusivity and partitioning of the acid and base. Only when a compound is used that is both water-and oil-soluble will molecular transfer to the droplet occur. We demonstrate this approach by modulating the internal pH of aqueous droplets (emulsion and in microfluidic structures) in oil. The advantage of our method compared to active strategies such as electrolysis is its technical simplicity. Further, the change in the pH does not lead to changes in the droplet volume. Therefore, our method does not suffer from the side effects of droplet merging strategies.

In **chapter 4,** we use the technology described in chapter 3 to develop a droplet-based viral fusion assay. We apply this platform to two influenza viruses that use very different entry mechanisms namely ganglioside (gd1a) and protein (glycophorin A) mediated fusion. This approach is however general and can be used to study other similar viruses such as HCV and dengue. Our designed platform can be used for binding as well as kinetic studies. We show that both influenza viral particles remain functional within the microdroplets. To demonstrate the applicability of our platform to conduct kinetic analysis, we performed a fusion kinetic analysis on one of these viruses and studied the dependency of the fusion rate constant on

acidity. The estimated fusion rate constants from the droplet-based assay were found to be in agreement with previous reports. Overall this chapter provides proof-of-concept of our viral assay design.

In the last experimental chapter of this thesis (**chapter 5**), we use the approach described in the previous chapter to design a microfluidic platform capable of high-throughput applications. Performing a high-throughput analysis of fast reactions, such as viral fusion assay, is a challenging task, which is addressed in this chapter by presenting a simple and compact opto-fluidic setup. By combining a sensitive multicolor fluorescence microscopy technique with a high-precision droplet-based microfluidic setup, we have developed a system that has a large field of view and thus is able to visualize the progression of a large number of droplets on a large microfluidic chip. By using an on-chip chemical exchange approach, we are able to change the content of the droplets and trigger the desired reaction. The large field of view allows for monitoring the content of many dynamic droplets without the need for storing them in a static, immobilized state. The technique presented allows for high-throughput studies of the kinetics of pH- or initiator-triggered reactions.

The results described in this thesis can be used as a basis to screen for inhibitors of influenza fusion. The obtained results demonstrate that the droplet-based system has the potential to provide the same level of quantitative information as that from standard virology techniques at a lower cost. Moreover, these techniques can be used to perform fluorescence based viral fusion assay on other enveloped viruses such as dengue and HCV. The functioning of these viruses can be investigated by monitoring the kinetics of viral fusion in such platforms. Furthermore, one can use such platform to screen for potential drugs that inhibit the fusion of the virus with a host membrane and test various doses of the inhibitor to extract information regarding inhibition efficiency.

One can speculate about the possible future extension of the proposed technology, beyond its application in anti-viral drug screening. In view of recent developments in single-cell analysis and sorting, we envision that our assay can be further expanded to study single-cell interactions with single viral particles. This will allow the study of not only binding and fusion events but also the biological processes that follow infection, such as changes in gene expression profile. Furthermore, viruses are commonly used as gene delivery vesicles for therapeutic purposes and engineering applications. An example of the latter is viral barcoding of cells. Our droplet-based viral infection assay platform can be used for controlled viral barcoding of cells, which could pave the way for studying cellular heterogeneity. Viral

genetic barcoding can be used to track single cells in heterogeneous populations such as differentiating hematopoietic stem cells and growing tumors.

Samenvatting en perspectief

Het influenzavirus wordt beschouwd als een grote bedreiging voor de volksgezondheid. Het verspreidt zich over de wereld in een seizoencyclus en een pandemie zou kunnen ontstaan door het hoge genetische aanpassingsvermogen van het virus. Met als doel om nieuwe anti-virale medicijnen snel en “high-throughput” te screenen, beschrijf ik in dit proefschrift een combinatie van microfluidics, met microdruppels als reactiekamers, met fluorescentie imaging technieken om een “lab-on-a-chip” platform te ontwikkelen, welke het mogelijk maakt om virale fusies onder verschillende omstandigheden te monitoren en de fusie kinetiek te bepalen. In dit proefschrift staat beschreven dat deze microdruppels kunnen worden gegenereerd door het printen van microdruppels arrays op een glazen drager of door een emulsie druppel systeem. In elke microdruppel kan virale fusie worden veroorzaakt door verlagen van de pH in de reactiekamer, wat lijkt op de condities die het virus in endosomen treft. Dit proefschrift presenteert nieuwe ontwikkelingen in de “on-chip” chemie in water, met toepassingen in virusdetectie en neutralisatie assays.

In hoofdstuk 1 geef ik achtergrond en het bredere academische kader door de recente vorderingen te beschrijven van de toepassing van druppel microfluidic-chip gebaseerde technieken in de bio-engineering, biochemie, chemische synthese en nanotechnologie. We behandelen onderwerpen zoals single-cell analyse, kleinschalige celculturen, “in-droplet” chemische synthese, “high-throughput” screenen van geneesmiddelen en de fabricage van nano apparaten. Daarnaast bespreek ik de opkomende onderzoeksrichtingen en, in het bijzonder, stel ik benaderingen voor voor toekomstig gebruik van de druppel-microfluidics in virologie.

Arrays van ondersteunde lipide bilagen (SLB) hebben een enorm potentieel in de ontwikkeling van geneesmiddelen en gemultiplext biochemisch onderzoek, maar ze zijn moeilijk te prepareren voor studie van virale fusies omdat het fusieproces een relatief complexe bilaag samenstelling vergt, evenals voldoende ruimte tussen de bilaag en de drager. Het maken van ondersteunde lipide bilagen met een biologisch relevante samenstelling, waaronder transmembraaneiwitten en diverse klassen van lipiden, is een significante uitdaging. De hoge mobiliteit van membraancomponenten, wat gewaarborgd wordt met het

voorgestelde platform, is zeer belangrijk in processen zoals virale fusie waar mobiliteit en structurele herschikkingen van membraaneiwitten nodig is. In **hoofdstuk 2** van dit proefschrift beschrijven we een generieke en gemakkelijke benadering voor de productie van vloeibare polymeer ondersteunde lipide bilagen dat zorgt voor de opname van een grote verscheidenheid aan lipiden en transmembraaneiwitten. De methode is gebaseerd op de vorming van een polymere borstel die lipide groepen presenteerd, gevolgd door het spin-coaten van membraanlipiden. Vervolgens worden transmembraaneiwitten opgenomen door de fusie van proteoliposomen met de bilaag. Daarnaast worden ook verschillende toepassingen van de technologie beschreven; deze omvatten het single-molecule tracken van de opgenomen transmembraaneiwitten in een bilaag, het visualiseren van de fusie van afzonderlijke, membraan omhulde virussen met een ondersteunde membraan en het testen van antilichaam inhibitie. Onze resultaten suggereren dat de eigenschappen van het membraan overeen komen met de eigenschappen in fysiologisch relevante omstandigheden. Dit toont de brede toepasbaarheid van onze benadering van de synthetische biologie, die gebruikt maakt van lab-on-a-chip technologie gecombineerd met biofysische en farmaceutische studies. Als voorbeeld gebruikten we dit platform om een single-particle virale fusie-assay uit te voeren op een chip, en deze technologie werd vervolgens toegepast om de effectiviteit van antivirale antilichamen te kwantificeren.

In **hoofdstuk 3** adresseren we een grote uitdaging voor de ontwikkeling van de droplet-based virale fusie assays: het regelen van de zuurgraad van de druppel, onafhankelijk van de concentraties van andere reagentia. Deze uitdaging komt veel voor in druppel gebaseerde chemie: hoe nieuwe reagentia te introduceren in een waterige druppel die zich in de oliefase bevind. De voorgestelde aanpak berust op het gebruik van reactanten (in ons geval een zuur) die oplosbaar zijn in zowel de waterige- als oliefase. Centraal in onze microfluïdische benadering zijn de oplosbaarheid, diffusie en verdeling van het zuur en de base. Alleen wanneer een verbinding oplosbaar is in zowel water als olie, zal moleculaire overdracht naar de druppel plaatsvinden. We tonen deze aanpak door het moduleren van de interne pH van de waterige druppeltjes (emulsie en in microfluïdische structuren) in olie. Het voordeel van onze werkwijze ten opzichte van actieve strategieën, zoals elektrolyse, is de technische eenvoud. Verder leidt de verandering van de pH niet tot veranderingen in de volume van de druppel. Daarom heeft onze werkwijze geen last van de bijwerkingen van de druppel samenvoeg strategieën.

In **hoofdstuk 4** maken we gebruik van de in hoofdstuk 3 beschreven technologie om een druppel gebaseerde virale fusietest te ontwikkelen. We passen dit platform toe op twee

griepvirussen die heel andere binnenkomst mechanismen gebruiken, namelijk ganglioside (gd1a) en eiwit (glycoforine A) gemedieerde fusie. Deze benadering is algemeen en kan worden gebruikt om andere soortgelijke virussen zoals HCV en dengue te bestuderen. Ons ontworpen platform kan worden gebruikt voor zowel bindings als kinetische studies. We tonen aan dat zowel influenza als virusdeeltjes functioneel blijven binnen de microdruppels. Om de toepasbaarheid van ons platform om kinetische analyse uit te voeren te tonen, hebben we een fusie kinetische analyse op een van deze virussen uitgevoerd en de afhankelijkheid van de fusie snelheidsconstante op de zuurgraad bestudeerd. De geschatte fusie snelheidsconstantes van de druppel-gebaseerde test bleken in overeenstemming te zijn met literatuur. Over het geheel geeft dit hoofdstuk een proof-of-concept van onze virale assay design.

In het laatste experimentele hoofdstuk van dit proefschrift (**hoofdstuk 5**) maken we gebruik van de in het vorige hoofdstuk beschreven benadering om een microfluïdische platform voor high-throughput toepassingen te ontwerpen. Het uitvoeren van een high-throughput analyse van snelle reacties, zoals virale fusie assay, is een uitdaging die geadresseerd wordt in dit hoofdstuk door de toepassing van een eenvoudige en compacte opto-vloeibare setup. Door het combineren van een gevoelig meerkleuren fluorescentie microscopie techniek met een hoge precisie droplet-gebaseerde microfluidic opstelling hebben we een systeem dat een groot gezichtsveld heeft en derhalve kan de progressie van een groot aantal druppels zichtbaar op grote microfluidic chip gevisualiseerd worden. Via een on-chip chemische uitwisseling benadering, kunnen we de inhoud van de druppeltjes veranderen en de gewenste reactie uitlokken. Het grote gezichtsveld staat het monitoren toe van de inhoud van vele dynamische druppeltjes zonder dat opslag in een statische, geïmmobiliseerde toestand nodig is. De gepresenteerde techniek maakt hoge-doorvoer onderzoek van de kinetiek van pH- of initiator geactiveerde reacties mogelijk.

De in dit proefschrift beschreven resultaten kunnen worden gebruikt als basis voor het screenen op remmers van influenza fusie. De verkregen resultaten tonen aan dat het druppel systeem het potentieel heeft om hetzelfde niveau van kwantitatieve informatie te verschaffen als die van standaard virologische technieken maar tegen lagere kosten. Bovendien kunnen deze technieken worden gebruikt om een fluorescentie gebaseerde virale fusie-test uit te voeren op andere omhulde virussen zoals dengue en HCV. De werking van deze virussen kan worden onderzocht door het monitoren van de kinetiek van virale fusie in deze platforms. Bovendien kan men een dergelijk platform gebruiken om te screenen op potentiële

geneesmiddelen die de fusie van het virus met tal van membranen remmen, en het testen van verschillende doses van de remmer om informatie over remming efficiëntie te verkrijgen.

Men kan speculeren over de mogelijke toekomstige uitbreiding van de voorgestelde technologie, buiten de toepassing in de anti-virale drug discovery. Gezien de recente ontwikkelingen in eencellige analyse en sortering, voorzien we dat onze test verder kan worden uitgebreid tot het besuderen van één cel interacties met enkele virale deeltjes. Dit zal de studie van niet alleen bindings- en fusiegebeurtenissen mogelijk maken, maar ook de biologische processen die volgen op infectie, zoals bijvoorbeeld veranderingen in genexpressie profiel. Verder worden virussen vaak gebruikt als genafgifte vesicles voor therapeutische doeleinden en technische toepassingen. Een voorbeeld van het laatste is virale barcoding cellen. Onze druppel gebaseerde virale infectie assay platform kan worden gebruikt voor het gecontroleerd viraal barcoden van cellen, die de weg voor het bestuderen van cellulaire heterogeniteit kunnen vrijmaken. Virale genetische barcodes kunnen worden gebruikt om individuele cellen in heterogene populaties te volgen zoals differentiatie hematopoietische stamcellen en groeiende tumoren.

Curriculum Vitae

I was born in Babol, a city located in the north of Iran. I did my middle school and high school education in Special School for Exceptional Talents. After graduation in 2002, I got enrolled in Mechanical Engineering undergraduate programme at the Noshirvani Technical University in Babol. During my undergraduate studies I did my engineering project in the group of prof. dr. Stefan Diez on the "Characterization of Microtubule Rotations During Cargo Transport" at Max Planck Institute (MPI-CBG) in Dresden, Germany. In 2006, I won IAESTE scholarship to join the Nano-optics group led by prof. dr. Vahid Sandoghdar at ETH Zurich where I did my undergraduate thesis on "Optical Detection and Contact Mechanics of Nano-Micro Particles on Biological and Engineered Surfaces". Right after my graduation, I was fortunate to receive an offer and a generous financial support from the Nanoscience center at the University of Jyväskylä in Finland to do my masters studies in nanoscience. While studying in Finland, I did a short internship in the Spectroscopy group of prof. dr. Janne Ihalainen. I then moved to the Netherlands to do my masters project on single molecule interrogation of proteins using optical tweezers in the group of prof. dr. Sander Tans. I completed my studies with a one-year Masters thesis on "Chirality-Controlled Preparation and Single Molecule Characterization of Carbon Nanotubes" in the nanoelectronics group of prof. dr. Markus Ahlskog at the University of Jyväskylä.

In August 2011 I started my PhD-project on droplet-microfluidics under the supervision of prof. dr. Antoine van Oijen in the single molecule biophysics group. My PhD project was concerned with developing a droplet-based microfluidic platform for on-chip viral-fusion studies. In the final stage of the PhD project, I joined the lab of prof. dr. David Weitz at Harvard University, where I further developed my skills in microfluidics. The results of the research conducted in the period between August 2011 and June 2015 are described in this thesis. These results include a novel technique for controlling droplet content based on biphasic diffusive exchange reagents. During my PhD, I worked as a TA for the courses in Mechanics (Statics and Mechanics of Materials), Modern Microscopy, and Thermodynamics. Moreover, I supervised two top Masters students in the context of their master theses.

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Epilogue and acknowledgements

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